EXPRESS MAIL CERTIFICATE

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_____ _____
Name (Print) Signature

Docket No.: 00630/100G184-US1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No. **32801**

In re Patent Application of:
Petanceska, Suzana; Gandy, Sam; and Frail, Donald E.

Application No.: 09/695,446 Art Unit: 1615

Filed: October 24, 2000 Examiner: L. Di Nola-Baron

For: METHODS OF IDENTIFYING AND USING AMYLOID-INHIBITORY COMPOUNDS

DECLARATION UNDER 37 C.F.R. §1.131

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

We, Suzana Petanceska, Sam Gandy, and Donald E. Frail, do hereby declare:

1. We, Sam Gandy and Donald E. Frail are citizens of the United States of America. I, Suzana Petanceska am a citizen of Macedonia. We are all more than 21 years of age.

2. We are the inventors of the subject matter described and claimed in the above-captioned application.

3. We are aware of, and have read U.S. Patent No. 6,524,616, issued to Notelivitz, et al. ("Notelivitz") on February 25, 2003, and claiming priority to provisional application 60/141,189, filed on June 25, 1999. We understand that the Examiner has relied upon this reference in the Office Action dated February 10, 2004 in rejecting claims 1-6, 20-25, and 31-33 under 35 U.S.C. §102(e).

4. Prior to June 25, 1999, the invention as claimed in Claims 1-6, 20-25, and 31-33 of the above-identified application was conceived and reduced to practice by us, on our behalf, or under our supervision and control, in the United States. Exhibits 1 to 12 providing evidence of the conception and reduction to practice of claims 1-6, 20-25, and 31-33 are described in the following paragraphs.

5. Prior to June 25, 1999, we conceived and reduced to practice a method for reducing a level of amyloid beta ("Aβ") peptides *in vivo* in accordance with Claims 1-6 and 31. For example, as shown in Exhibit 1, we tested three experimental sets of guinea pigs: ovariectomized animals ("ovx") (e.g., "control animals"), ovx animals that received a low-dose, 1 mg/kg 17β-estradiol ("E2") treatment ("ovx + low-dose E2"), and ovx animals that received a high-dose, 5 mg/kg E2 treatment ("ovx + high-dose E2"). The animals were ovariectomized and weighed prior to the beginning of the treatment. Treatment began eight (8) weeks after ovariectomy. The ovx + E2 animals received E2 for ten (10) days. After treatment, all of the animals were sacrificed and trunk blood was collected for determination of E2 levels in the serum by radio-immunoassay. Uteri were removed and weighed to establish estradiol-induced uterine hypertrophy. Data of the pre-treatment weight of the animals (Exhibit 1, col. 2), the type of treatment received for

10 days (Exhibit 1, col. 3), and the corresponding uterine weight of the sacrificed animals (Exhibit 1, col. 1) is listed on the date-redacted lab notebook pages attached hereto in Exhibit 1. Each of the redacted dates in Exhibit 1, i.e., the date of ovariectomy and the dates of E2 treatment, are prior to June 25, 1999.

6. Prior to June 25, 1999, our tests established that prolonged ovariectomy resulted in uterine atrophy, while the 10 day oral administration of E2 led to uterine hypertrophy in ovx guinea pigs. Evidence of the uterine atrophy and hypertrophy can be found in the ratio of uterine weight to body weight we recorded for this experiment on the lab notebook page attached hereto in Exhibit 2.

7. Prior to June 25, 1999, our tests further established that prolonged ovariectomy resulted in decreased serum E2 levels, while E2 treatment of ovx animals led to a dose-dependent increase in serum E2 levels, as recorded on the lab notebook page attached hereto in Exhibit 3, page 3. Guinea pigs numbered 1 and 2 were ovx control animals receiving no E2 treatment, guinea pigs numbered 3, 4, 5, and 9 were ovx plus 1 mg/kg low-dose E2, and guinea pigs numbered 8 and 12 were ovx plus 5 mg/kg high-dose E2 as recorded on the date redacted lab notebook page attached hereto in Exhibit 1. Accordingly, our experimental data in Exhibit 3, page 3 shows that ovx animals receiving both the low-dose E2 treatment (e.g., guinea pigs numbered 3, 4, 5, and 9), and high-dose E2 treatment (e.g., guinea pigs numbered 8 and 12), led to a dose dependent increase in serum E2 levels; while ovx control animals receiving no E2 treatment (e.g., guinea pigs numbered 1 and 2) had decreased serum E2 levels. The data in Exhibit 3, page 3 was included in a three-page facsimile sent prior to June 25, 1999. Irrelevant portions of this

facsimile have been redacted. Exhibit 3, page 1 is a date-redacted facsimile cover page enclosing the data in page 3 referred to herein. The redacted date on all pages is prior to June 25, 1999.

8. Prior to June 25, 1999, we assessed brain amyloid precursor protein, amyloid peptide β 1-40 ("Aβ40") and amyloid peptide β 1-42 ("Aβ42") levels in ovx control animals and ovx animals receiving E2 treatment (ovx + low-dose E2 and ovx + high-dose E2) following administration of E2 using Aβ40 and Aβ42-specific ELISA assays. Normalized values of total brain protein (Exhibit 4, page 1), total brain Aβ (Exhibit 4, page 2), brain Aβ-40 (Exhibit 4, pages 3 and 4), and brain Aβ-42 (Exhibit 4, pages 5 and 6) levels are recorded in the date-redacted lab notebook pages attached hereto as Exhibit 4. Each of the redacted dates are prior to June 25, 1999.

9. Prior to June 25, 1999, our Aβ-40 and Aβ-42-specific assays established a pronounced increase in brain Aβ-40 and Aβ-42 levels, and total Aβ levels after ovx. Compared to the ovx control animals, E2 treatment of ovx animals was associated with a *reduction* in the amount of Aβ-40 and Aβ-42, and total Aβ levels. We recorded data from these tests on the date-redacted lab notebook pages attached hereto in Exhibit 5. Each of the redacted dates are prior to June 25, 1999.

10. Prior to June 25, 1999, our experiment showed that oxv plus E2 treatment at both dosage levels significantly reversed the ovariectomy-induced increase in brain Aβ levels. High-dose E2 treatment did not lead to a further decrease in brain Aβ beyond that observed with the low-dose E2 treatment. We recorded these results on the date-redacted lab notebook pages attached hereto in Exhibit 6. Each of the redacted dates are prior to June

25, 1999.

11. Prior to June 25, 1999, our results further show that soluble APPα ("sAPPα") levels, a metabolite of amyloid precursor protein ("APP"), did not change in response to ovx plus E2 treatment. We recorded these results in a scattergraph on the lab notebook page attached hereto in Exhibit 7, and in a bargraph on the lab notebook page attached hereto in Exhibit 8. For purposes of testing, the sAPPα level was normalized to full length APP ("flAPP" or "hAPP"). The data for normalized levels of sAPPα are recorded on the lab notebook pages attached hereto in Exhibit 9. Although the data we recorded in the lab notebook pages attached hereto as Exhibits 7-9 is not dated, it is based on the document (attached hereto as Exhibit 10), and the draft document (attached hereto as Exhibit 11), both dated prior to the June 25, 1999 date of Notelivitz. The title and date noted on the documents attached hereto as Exhibits 10 and 11 have been redacted. These documents cite to the graph attached hereto in Exhibits 7 as Fig. 5A, and the graph attached hereto in Exhibit 8 as Fig. 5B. The documents also include a reference that the values for each sample were normalized to the values obtained for flAPP as recorded in Exhibit 9.

12. Prior to June 25, 1999, we had in our possession a method for reducing a level of Aβ peptides *in vivo*, by administering an Aβ level reducing dose of an estrogen compound to an animal, wherein the animal without the estrogen compound treatment has an increased level of Aβ and wherein the dose of the estrogen compound does not affect soluble APP levels, in accordance with Claims 1-6, and 31. Our ovx guinea pig experimental model data shown in the exhibits described herein (e.g., Exhibits 1-12), demonstrates possession of this method.

13. Prior to June 25, 1999, we had in our possession a method for delaying or reducing the likelihood of, or ameliorating, a disease or disorder associated with amyloidosis, which method comprises administering an Aβ level reducing dose of an estrogen compound to a subject who has an increased risk for developing or shows a symptom of the disease or disorder associated with amyloidosis, wherein the dose of the estrogen compound does not affect soluble APP levels, in accordance with Claims 20-26 and 32-33. Our results of the testing on guinea pigs as shown in the exhibits described herein (e.g., Exhibits 1-12), further demonstrate possession of this method.

14. It is well known by persons having ordinary skill in the art that animal models can be used to determine the pharmacology of Alzheimer's disease (AD). AD is characterized by the accumulation of, *inter alia*, amyloid plaques and deposits, of which Aβ is a major component (See specification of the '466 application, p. 1, lines 11-20). Aβ is derived by proteolytic processing of APP. *Id.* Guinea pigs are a useful animal model because their endogenous amino acid sequence of the Aβ peptide is identical to the human sequence. *See* Johnstone, et al., "Conservation of the sequence of the Alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis," Mol. Brain Res. 1991 Jul.; 10(4): 299-305 at 303 (Exhibit 12). Certain transgenic animals are also useful animal models because after introduction of a transgene, they too will express an amino acid sequence of the Aβ peptide identical to the human sequence. Accordingly, the testing described herein supports use of the claimed invention in humans.

15. We declare that statements made in this Declaration are of our own knowledge and are true and that all statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 08/09/04

Suzana Petanceska

Date: _____

Sam Gandy

Date: _____

Donald E. Frail

15. We declare that statements made in this Declaration are of our own knowledge and are true and that all statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date:_____

Susanna Petanceska

Date: 7/30/04

Sam Gandy

Date:_____

Donald E. Frail

15. We declare that statements made in this Declaration are of our own knowledge and are true and that all statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date:_____

Susanna Petanceska

Date:_____

Sam Gandy

Date: July 30, 2004

Donald E. Frail

	uterus	weight	treatment
(1)	0.350	690	OVX
(2)	0.900	330g	OVX
(3)	2.400	620	OVX + 1mg/kg
(4)	2.252	620	OVX + 1mg/kg
(5)	2.682	600	OVX + 1mg/kg
(6)	0.435	530	OVX
(8)	1.7640	440	OVX + 5mg/kg
(9)	2.180	645	OVX + 1mg/kg
(12)	2.100	370	OVX + 5mg/kg

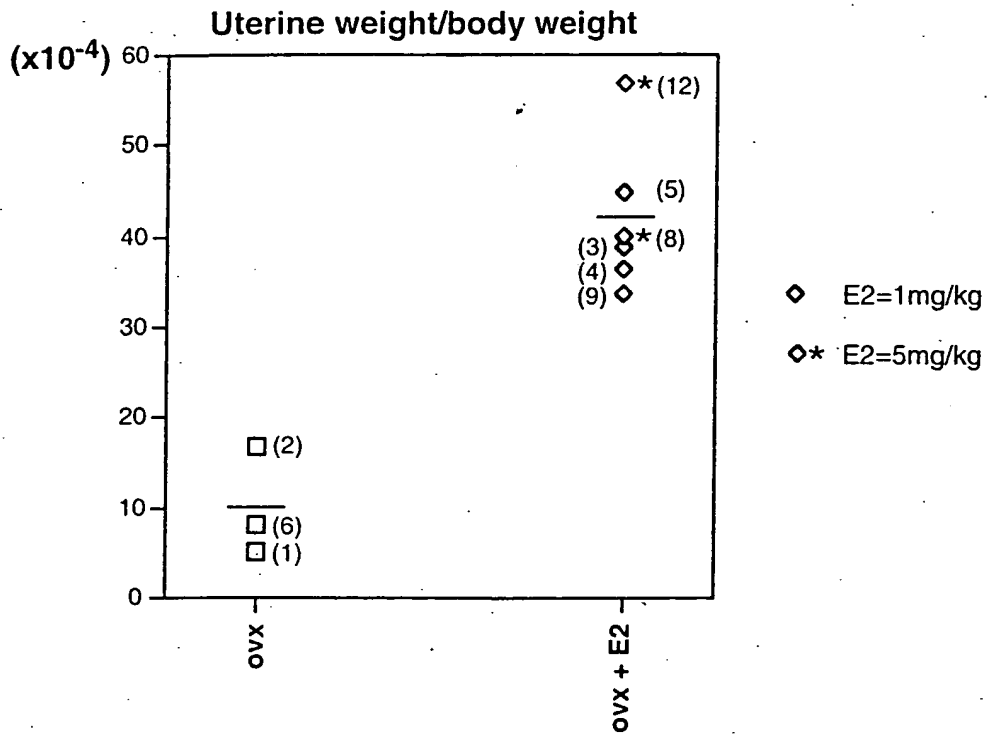
Guinea pigs

(OVX . REDACTED

E2 - 10 days

REDACTED

(09 Fuhrmann)


Uterine weight/body weight
(x10-4)
60
50
40
30
20
10
0
(12)
(5)
(3)
(8)
(4)
(9)
(2)
(6)
(1)
ovx
ovx + E2
E2=1mg/kg
E2=5mg/kg

WYETH AYERST

WOMEN'S HEALTH RESEARCH INSTITUTE

FACSIMILE TRANSMISSION
WYETH-AYERST RESEARCH
WOMEN'S HEALTH RESEARCH INSTITUTE
145 KING OF PRUSSIA ROAD
RADNOR, PA 19087

DATE: REDACTED

TO: Suzana c/o Sam Gandy PHONE #: 914-398-5436

COMPANY/LOCATION: Nathan Kline

FAX #: 914-398-5422

NUMBER OF PAGES (Including cover page): 3

FROM: Dan Frail PHONE #: 610 341 2860

LOCATION:

FAX: (610) 989-4832

COMMENTS: Finally! Data Attached.

REDACTED

REDACTED

Dan

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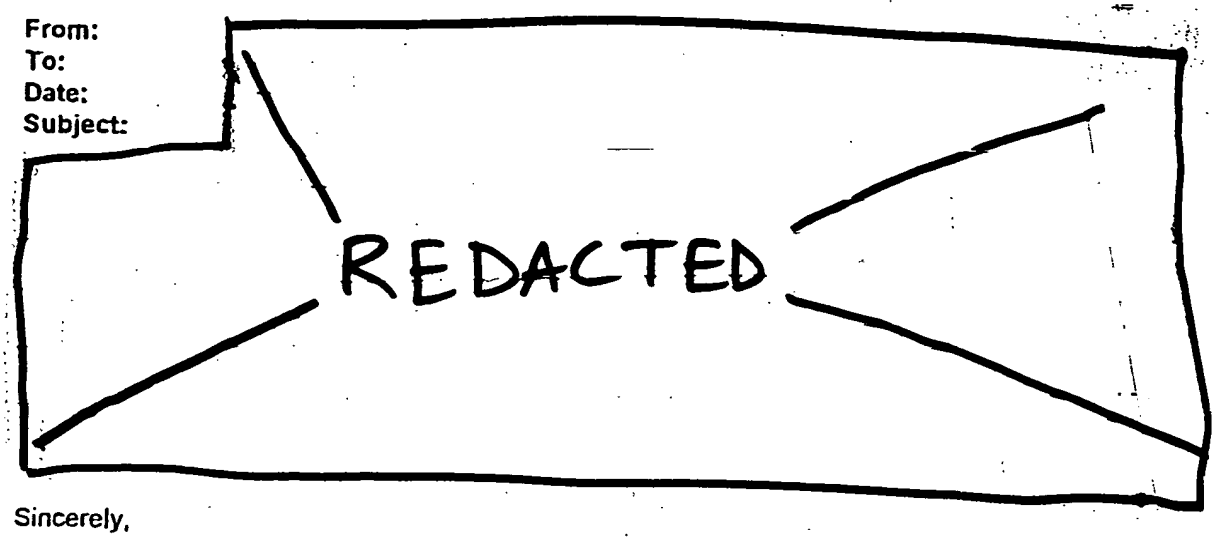
From:
To:
Date:
Subject:



Sincerely,

REDACTED

cc: REDACTED

File:g\cnylab\reflab\wyeth\pig99.xls

Rat plasma for Estradiol from Wyeth-Ayerst

R&D bldg: #145
Brian Arey
Tel: (610) 688 - 4422 ext: 2720
FAX: (610) 989 - 4832

Date faxed03/29/99

#	Sample ID	Estradiol(pg/mL)	
1	GP 1	23	1
2	GP 2	102	2
3	GP 3	748	3
4	GP 4	1592	4
5	GP 5	491	5
6	GP 6	66	6
7	GP 8	16100	8
8	GP 9	1142	9
9	GP 12	14000	12

C = 50 ±

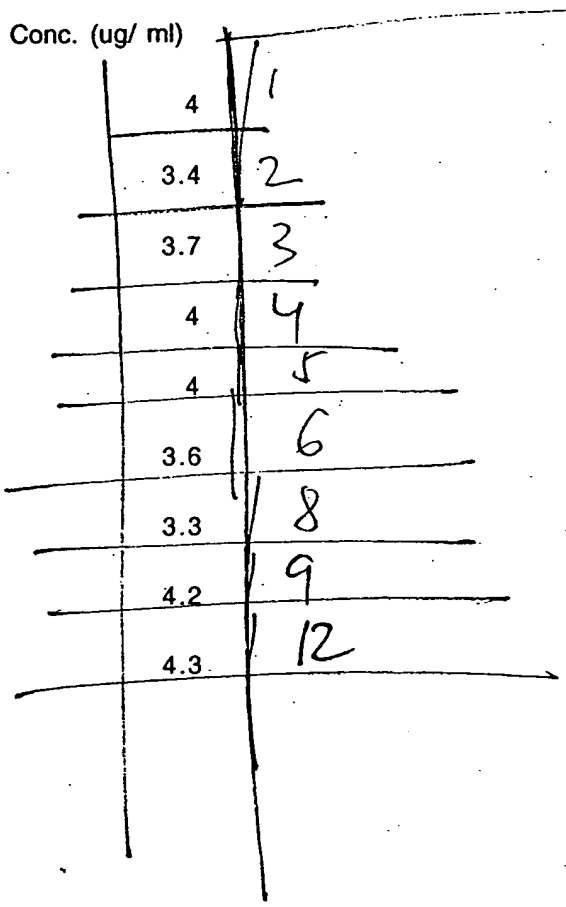
1 ng/L = 1000 ±

5 ng/L = 15000 ±

REDACTED GP1 BCA extracts

Pig #

Guinea Pig; DEA	ABS. (25 ul of samp. in 100 ul =4x dil.)	raw conc.	act. Conc. (ug/ ml)
1	1.217	980.69	
	1.237	999.98	
2	1.101	869.35	
	1.046	816.6	
3	1.231	994.49	
	1.066	835.67	
4	1.189	953.79	
	1.264	1025.9	
5	1.221	984.66	
	1.275	1035.9	
6	1.389	1145.2	
	0.851	630.42	
8	1.037	808.54	
	1.081	850.29	
9	1.257	1018.7	
	1.302	1061.9	
12	1.283	1044.3	
	1.343	1101.4	



* NOTE: Loaded 100 ug of each sample to two 10 % gels. Gels labeled GP1A and GP1B.

GP1A : α GE10 (1:1000) mouse

GP1B : α C1G1 (1:1000) mouse

GP FA BCA RESULTS REDACTED

GP number	ABS.	100ul volume	CONC.	AVG. CONC.	ACT. CONC.
1	0.468		159.26	161.09	0.16
	0.477		162.92		
2	0.488		166.98	166.41	0.17
	0.485		165.84		
3	0.644		225.36	233.05	0.23
	0.685		240.74		
4	0.571		197.79	204.27	0.2
	0.605		210.75		
5	0.646		226.09	222.78	0.22
	0.629		219.47		
6	0.724		255.03	250.49	0.25
	0.871		245.94		
8	0.852		310.26	306.56	0.31
	0.796		302.86		
9	0.772		282.05	277.67	0.28
	0.908		273.28		
12	0.893		323.95	321.12	0.32
	0.699		318.29		

NOTE: samples extracted with FA on REDACTED

Column1		Column2	
Mean	5.44	Mean	4.005
Standard Erroı	0.54720502	Standard Erroı	0.55549827
Median	5.9	Median	3.68
Mode	#N/A	Mode	#N/A
Standard Devi	0.9477869	Standard Devi	1.36068733
Sample Varian	0.8983	Sample Varian	1.85147
Kurtosis	#DIV/0!	Kurtosis	-1.8963531
Skewness	-1.6695725	Skewness	0.50730946
Range	1.72	Range	3.02
Minimum	4.35	Minimum	2.65
Maximum	6.07	Maximum	5.67
Sum	16.32	Sum	24.03
Count	3	Count	6

MSTC
369

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	5.44	4.005
Variance	0.8983	1.85147
Observations	3	6
Pooled Varianc	1.57913571	
Hypothesized	0	
df	7	
t Stat	1.6149429	
P(T<=t) one-t	0.07517865	
t Critical one-t	1.89457751	
P(T<=t) two-t	0.15035729	
t Critical two-t	2.36462256	

$p \leq 0.075$

369

Column1		Column2	
Mean	3.80333333	Mean	2.88833333
Standard Erroı	0.63024687	Standard Erroı	0.35482781
Median	4.08	Median	3.285
Mode	#N/A	Mode	#N/A
Standard Devi	1.09161959	Standard Devi	0.86914709
Sample Varian	1.19163333	Sample Varian	0.75541667
Kurtosis	#DIV/0!	Kurtosis	-1.7740844
Skewness	-1.0672468	Skewness	-0.8582698
Range	2.13	Range	1.96
Minimum	2.6	Minimum	1.7
Maximum	4.73	Maximum	3.66
Sum	11.41	Sum	17.33
Count	3	Count	6

AB42
369

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	3.80333333	2.88833333
Variance	1.19163333	0.75541667
Observations	3	6
Pooled Varianc	0.88005	
Hypothesized	0	
df	7	
t Stat	1.37937522	
P(T<=t) one-t	0.10511689	
t Critical one-t	1.89457751	
P(T<=t) two-t	0.21023377	
t Critical two-t	2.36462256	

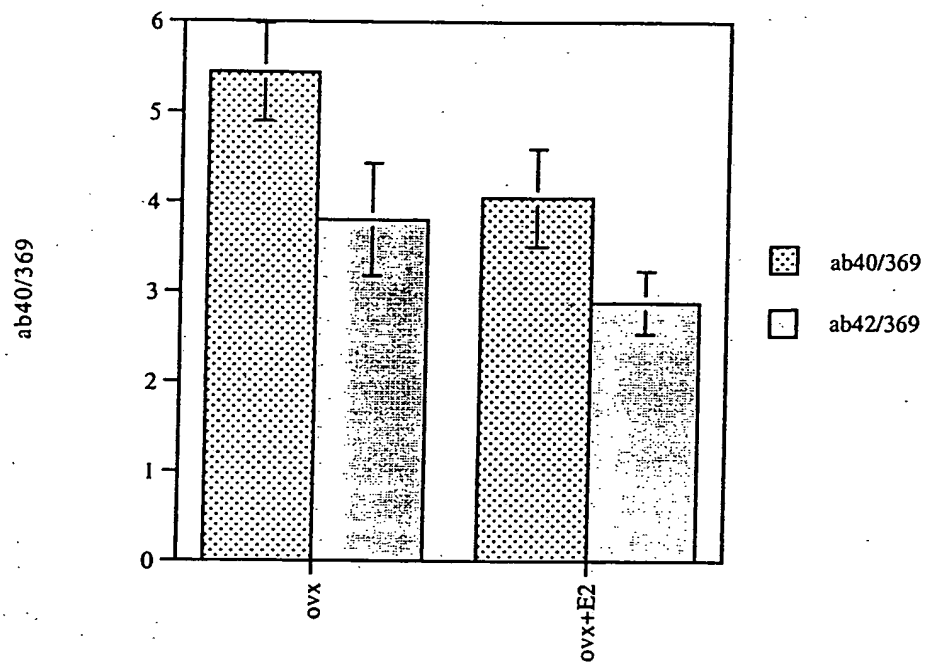
$p \leq 0.105$

Attention: Suzanne B. REDACTED

pg/ml

Sample No.	REDACTED	Aβ40	REDACTED	Aβ42	
GP 1	453	ONX	362	200 (1)	ONX
2	480	ONX	482	374 (2)	ONX
3	465	ONX+E2	486	266 (3)	ONX+E2
4	436	ONX+E2	434	257 (4)	ONX+E2
5	362	ONX+E2	548	347 (5)	ONX+E2
6	392	ONX	501	368 (6)	ONX
8	236	ONX+E2	485	167 (8)	ONX+E2
9	325	ONX	422	205 (9)	ONX
12	353	ONX+E2	437	353 (12)	ONX+E2
DB 1	1054	ONX	696	OX	424 ONX
2	>1312	sham	1225	sham	484 sham
3	823	ONX	494	OX	372 ONX
4	1174	ONX	865	OX	412 ONX
5	>1312	Cont	1161	Cont	386 Cont
6	1165	Cont	1284	Cont	438 Cont
7	>1312	ONX	1493	OX	544 ONX
DB Media		0		<25	
GP "		0		<25	

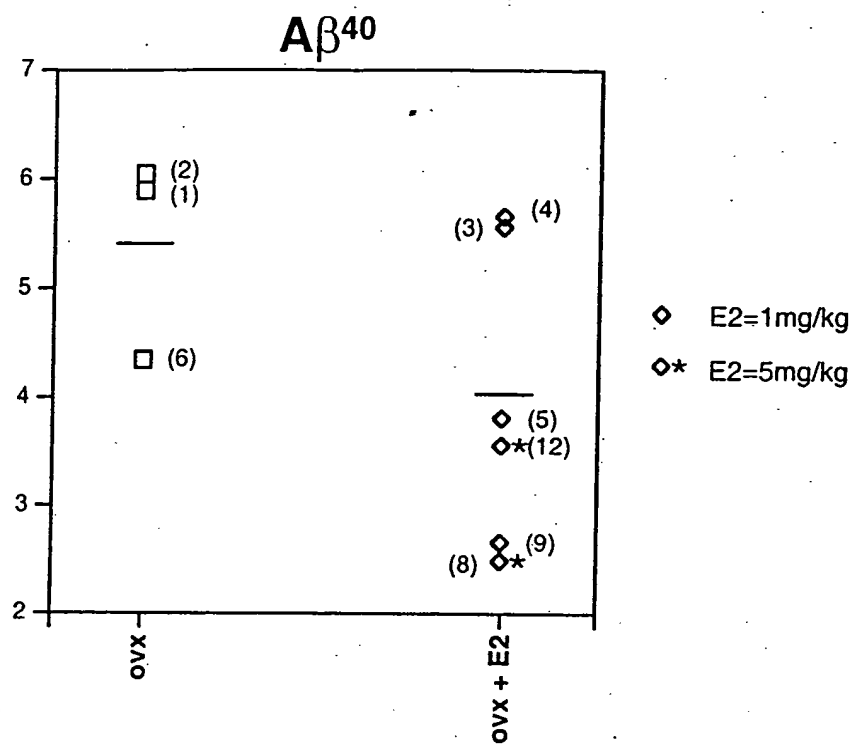
Data #2

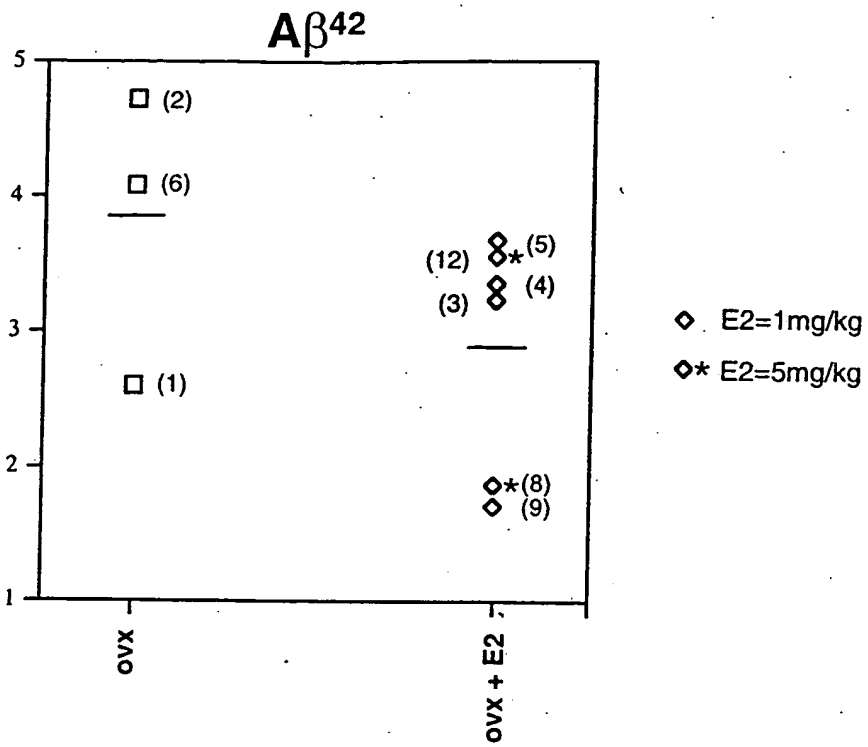


Column 2

Aβ40 — $p \leq 0.075$

Aβ42 - $p \leq 0.105$

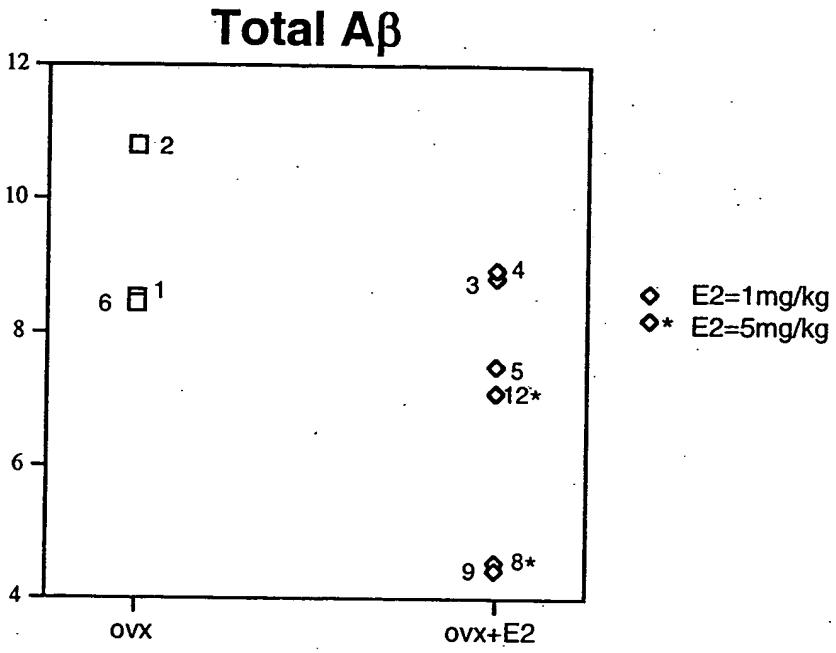

Aβ40
7
6
5
4
3
2
(2)
(1)
(6)
(3)
(4)
(5)
*(12)
(8)
*
(9)
OVX
OVX + E2
E2=1mg/kg
E2=5mg/kg


Aβ42
5
4
3
2
1
(2)
(6)
(1)
(12)
(5)
(4)
(3)
(8)
(9)
OVX
OVX + E2
E2=1mg/kg
E2=5mg/kg

Attention: Suzanne, B.

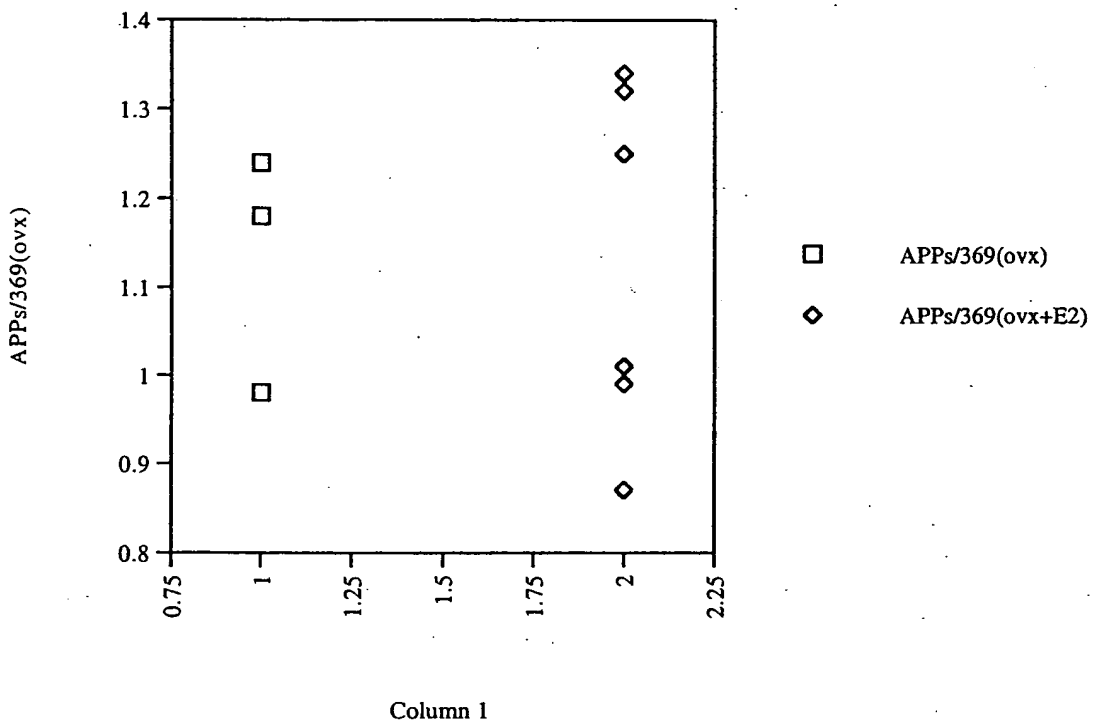
pg/ml

Sample No.	REDACTED	AB40	REDACTED	AB42	
GP 1	453	OVX	362	200 (1)	OVX
2	480	OVX	482	374 (2)	OVX
3	465	OVX+E2	486	266 (3)	OVX+E2
4	436	OVX+E2	434	257 (4)	OVX+E2
5	362	OVX+E2	548	347 (5)	OVX+E2
6	392	OVX	501	368 (6)	OVX
8	236	OVX+E2	485	167 (8)	OVX+E2
9	325	OVX+E2	422	205 (9)	OVX+E2
12	353	OVX+E2	437	353 (12)	OVX+E2
DB 1	1054	OVX	696	OX 424	OVX
2	>1312	sham	1225	sham 484	sham
3	823	OVX	494	OX 372	OVX
4	1174	OVX	865	OX 412	OVX
5	>1312	Cont	1161	Cont 386	Cont
6	1165	Cont	1284	Cont 438	Cont
7	>1312	OVX	1493	OX 544	OVX
DB Media		0		<25	
GB "		0		<25	


Total Aβ
12
10
8
6
4
2
6
1
3
4
5
12*
9
8*
ovx
ovx+E2
E2=1mg/kg
E2=5mg/kg

APPs2
369

Data #3



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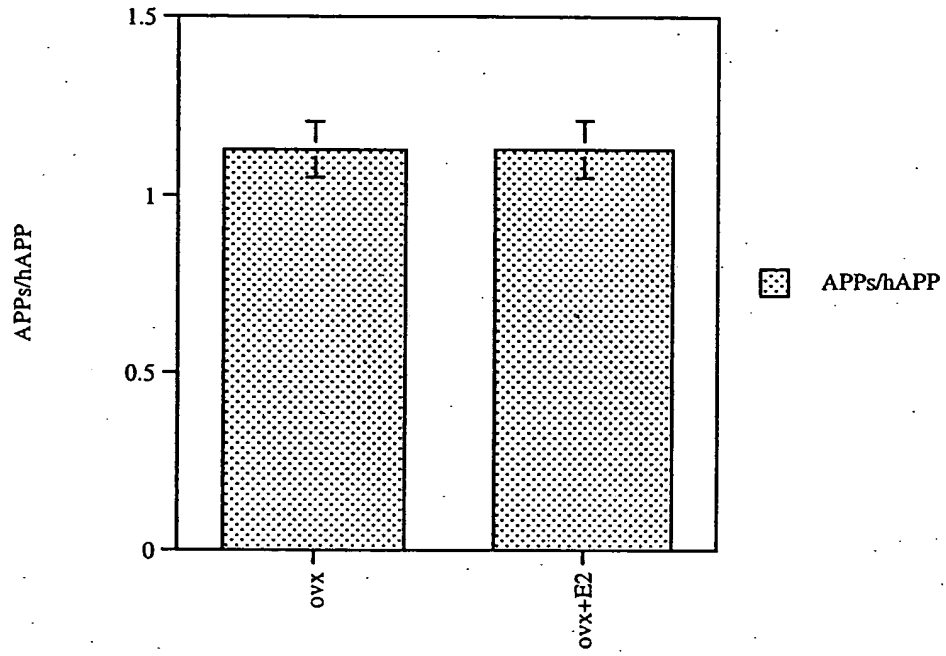


Column 2

$p \leq 0.50$

Apps

1 App

Column1		Column2	
Mean	1.13333333	Mean	1.13
Standard Erroı	0.07859884	Standard Erroı	0.08087027
Median	1.18	Median	1.13
Mode	#N/A	Mode	#N/A
Standard Devi	0.13613719	Standard Devi	0.19809089
Sample Varian	0.01853333	Sample Varian	0.03924
Kurtosis	#DIV/0!	Kurtosis	-2.3327707
Skewness	-1.3613014	Skewness	-0.1620978
Range	0.26	Range	0.47
Minimum	0.98	Minimum	0.87
Maximum	1.24	Maximum	1.34
Sum	3.4	Sum	6.78
Count	3	Count	6

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	1.13333333	1.13
Variance	0.01853333	0.03924
Observations	3	6
Pooled Varianc	0.03332381	
Hypothesized	0	
df	7	
t Stat	0.02582358	
P(T<=t) one-t	0.49005941	
t Critical one-t	1.89457751	
P(T<=t) two-t	0.98011881	
t Critical two-t	2.36462256	

$p \leq 0.50$

Redacted

Redacted

submitted by Donald E. Frail, Ph.D.

Women's Health Research Institute

Wyeth-Ayerst Research

DESCRIPTION

The amount of amyloid precursor protein, amyloid peptide β 1-40, and β-amyloid peptide 1-42 were measured in brain extracts of ovariectomized (Ovex) animals following administration of 17β-estradiol and control ovariectomized animals. While there was no difference in the amount of amyloid precursor protein between the two groups, the amount of amyloid β peptide 1-40 and amyloid β peptide 1-42 were reduced in those animals treated with estrogen compared to controls. The amyloid β peptides accumulate in the brains of patients with Alzheimer's disease and are toxic to neurons. Estrogens are therefore claimed as modulators of amyloid β peptide and would therefore have utility for the prevention and treatment of Alzheimer's disease. Furthermore, the animal model employed, including both ovex and intact animals, is claimed as an in-vivo assay for the identification and characterization of compounds that modulate the amount of amyloid β peptides present in brain, other organs, and biological fluids.

Abbreviations: AD, Alzheimer's disease, **Aβ,** amyloid beta; **APP,** amyloid precursor protein; **flAPP,** full-length APP; **sAPP,** soluble APP; **ovex,** ovariectomized; **E2,** 17β-estradiol; **BW,** body weight; **UW,** uterine weight; **p.o.,** per os; **DEA,** diethyl amine; **FA,** formic acid; **ELISA,** enzyme-linked immunosorbent assay.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the accumulation of senile plaques in the brain, which are formed as a result of deposition of potentially neurotoxic 40- and 42-amino acid amyloid β peptides (Aβ). Recent studies indicate that postmenopausal estrogen replacement therapy may prevent or delay the onset of AD (Tang et al., 1996). In addition, the Aβ peptides have been shown to be toxic to neurons in culture. There is evidence that physiological concentrations of 17-β estradiol (E2) reduce the release of Aβ peptides from primary neuronal cultures of rat and mouse origin, and from human embryonic cerebrocortical neurons (Xu et al., 1998), suggesting a mechanism by which estrogen replacement therapy may delay and/or prevent AD.

Here we describe and claim a methodology which enables screening of the effects of estrogen on the levels of Aβ peptides in brain *in vivo* using experimental animals. We also describe results which show that E2 can reduce the levels of Aβ peptides in brain *in vivo* and therefore claim the use of estrogens as modulators (reducers) of the amount of Aβ peptide in brain.

METHODS

Maintenance of animals and treatment. Nine ovariectomized female guinea pigs were purchased from Covance Research (Denver, PA); the animals were ~8 weeks old at the time of surgery. Throughout the study the animals were fed *ad libitum* in a controlled lighting environment (6 h light; 19 h dark cycles). During the first three weeks after surgery, the animals were fed standard lab chow (Purina, Richmond, IN); the standard lab chow was then replaced with powdered, casein-based, soy-free diet (catalogue # 5713M-O, Purina, Richmond, IN) to exclude the presence of phyto-steroids in the diet. After 5 weeks on soy-free diet the animals were divided into three experimental sets: i) ovx-only (n=3), ii) ovx+low E2 group, (n=4, 1mg E2/1kg BW), and iii) ovx+high E2 group, (n=2, 5mg of E2/1kg BW). 17-β estradiol (catalogue # E8875; Sigma, St. Louis, MO) was administered p.o. by powdering the hormone into the soy-free chow. Prior to the beginning of the treatment, the animals were weighed, and average daily food intake for each animal using this particular diet was determined in order to supplement the hormone at the desired concentration. The ovx+E2 animals received estradiol for 10 days.

Tissue collection. At the end of the treatment, animals were sacrificed by decapitation. Trunk blood was collected for determination of E2 levels in the serum by radio-immuno-assay. Uteri were removed and weighed to establish estradiol-induced uterine hypertrophy. The brains were immediately dissected; the cerebellum was removed from each brain, and the rest of the brain was divided in hemispheres, which were snap-frozen, and stored at -80 °C.

Preparation of brain extracts. Soluble proteins from the brains were recovered using a modification of a previously described protocol (Savage et al., 1998). The hemispheres were homogenized in 0.2% DEA/50 mM NaCl at 1:10 w/v ratio, by 5-6 strokes of a Dounce homogenizer. The DEA homogenate was centrifuged for 90 min at 100 000g. The DEA supernatants were neutralized to pH ~8.0 by addition of 1/10th vol. of 0.5M Tris-Cl pH 6.8, aliquoted and snap- frozen. The DEA pellets were homogenized in 70% formic acid, and

centrifuged for 1 h at 100 000g. The FA supernatants were neutralized to ~pH 8.0 with 1N NaOH and multiple aliquots of each FA extract were snap-frozen. The protein concentrations of the DEA and FA supernatants were determined using the BCA reagent assay kit (Pierce, Rockford, IL).

Detection of sAPPα, flAPP, Aβ40 and Aβ42.

1) sAPPα was detected by Western blotting of proteins from the DEA extracts using the monoclonal antibody 6E10 (Senetek, St. Loius, MO), which recognizes residues 5-10 from the Aβ region. The DEA extraction recovers soluble and not membrane embedded proteins; this was confirmed by probing duplicate Western blots which contained increasing amounts of proteins from DEA extracts (10μg-200μg), with either 6E10, or an antibody against the C-terminus of APP, C1.61 (courtesy of Dr. Paul Mathews, NKI) (Fig.1). Fig. 1 shows that soluble APP is the preponderant species in the DEA extract, while flAPP, albeit present, is a minor species.

For detection of the effect of E2 on the levels of sAPPα, we performed Western blotting of duplicate samples from the DEA extracts (50μg/lane) with 6E10, followed by enhanced chemiluminescence. Multiple exposures of the immunoblots were scanned using the ScanAnalysis software. The average values (in densitometric units) for each sample were then normalized to the values obtained for flAPP. Full-length APP levels were determined by immunoblotting of FA extracts (20μg/lane) with antibody 369 (made against the cytoplasmic tail of APP, residues 645-695) in duplicate, and utilizing densitometric analysis of multiple exposures of the immunoblots.

2) The levels of soluble Aβ40 and Aβ42 were determined by Aβ40- and Aβ42-specific ELISA assays (Mehta et al. 1998) in the laboratory of Dr. Pankaj Mehta (Institute for basic Research in Developmental Disabilities, Staten Island NY) on a fee-for-service basis.

RESULTS

Summary: Ten day oral administration of 17β estradiol led to uterine hypertrophy in ovariectomized guinea pigs. This treatment also resulted in a decrease in brain Aβ40 and Aβ42 levels, but did not alter the levels of sAPPα.

Orally administered 17β estradiol causes uterine hypertrophy. In the course of 10 days the animals received either soy-free diet, or soy-free diet supplemented with E2 (1mg E2/1kg BW, or 5mg E2/kg BW). The uteri of the ovx+E2 animals were hypertrophied, as shown on the scatter-graph in Fig. 2A. Comparing the uterine weight/body weight index for each animal showed the same effect of E2 on the uterine weight. Fig. 2B is a bar-graph representation of the same data.

17β estradiol treatment reduces the levels of soluble, brain Aβ peptides, *in vivo*. E2 treatment resulted in a similar reduction of the levels of soluble Aβ40 and Aβ42 peptide. The scatter-graphs in Fig.3 show the values for Aβ40 (panel A.), and Aβ42 (panel B.) (pg/ml) normalized to the levels of flAPP (densitometric units, DU) for individual animals. The bar-graph in Fig 4. depicts the mean values for Aβ40 and Aβ42 levels for both the ovx and ovx+E2 groups. The differences between the mean values of the ovx and ovx+E2 groups were 26.5% for Aβ40 and 24.3% for Aβ42. It is important to note that at E2 dose of 5mg/kg, the Aβ40 levels were diminished by 43.1%.

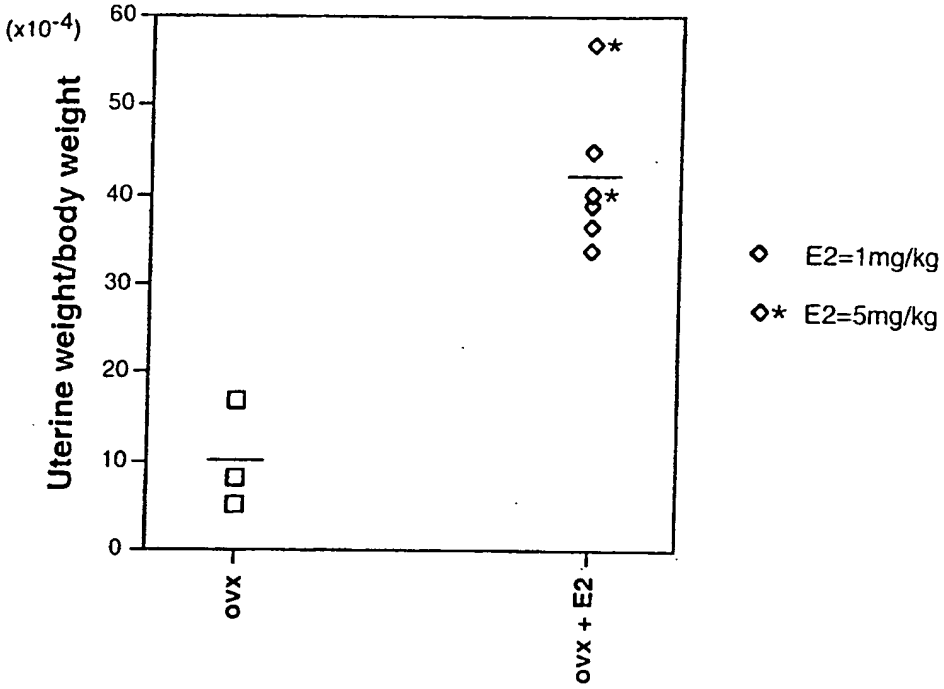
Estradiol treatment does not alter the levels of sAPPα *in vivo*. The levels of sAPPα (determined by quantitative Western blotting of extracts of soluble brain proteins) were not different between the ovx and ovx+E2 animals, as shown on the scatter-graph in Fig. 5A, and the bar-graph in Fig. 5B. The sAPPα values were normalized to the values for flAPP.

Figure 1


blotting antibody
10µg
50µg
100µg
200µg
6E10
C1.61
sAPPα
flAPP
DEA extract

Figure 2

A.


(x10-4)
Uterine weight/body weight
60
50
40
30
20
10
0
ovx
ovx + E2
E2=1mg/kg
E2=5mg/kg

B.

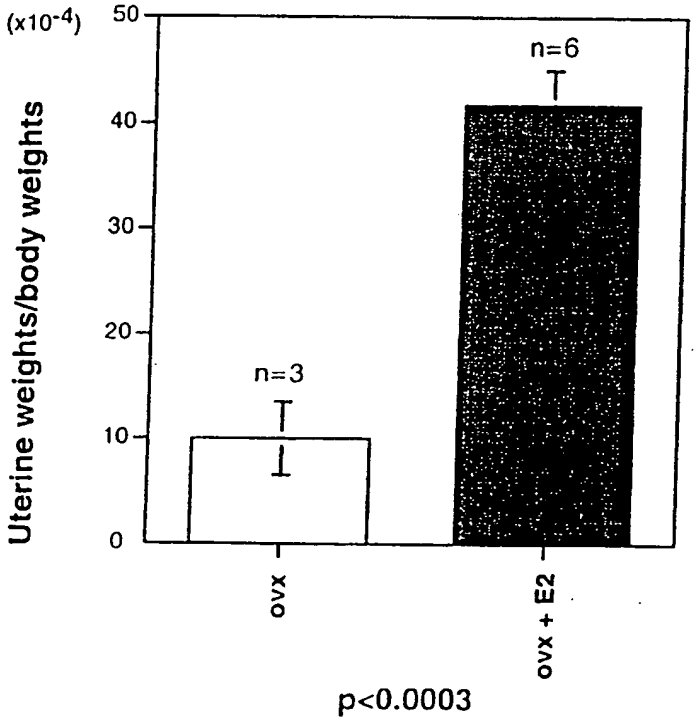
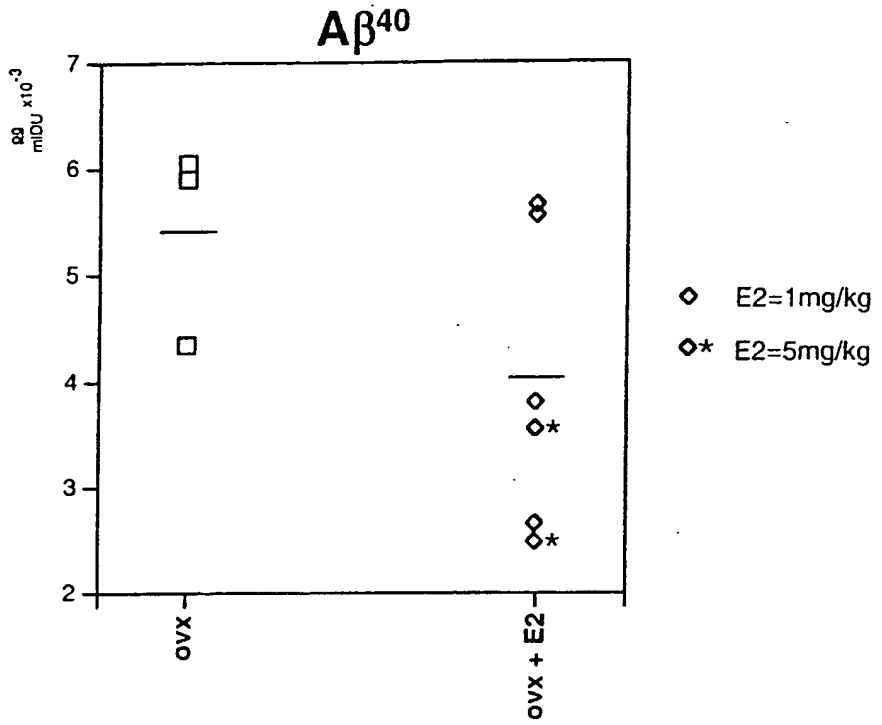

(x10-4)
Uterine weights/body weights
50
40
30
20
10
0
n=6
n=3
ovx
ovx + E2
p<0.0003

Figure 3

A.


Aβ40
ng mlDU x10-3
7
6
5
4
3
2
OVX
OVX + E2
E2=1mg/kg
E2=5mg/kg

B.

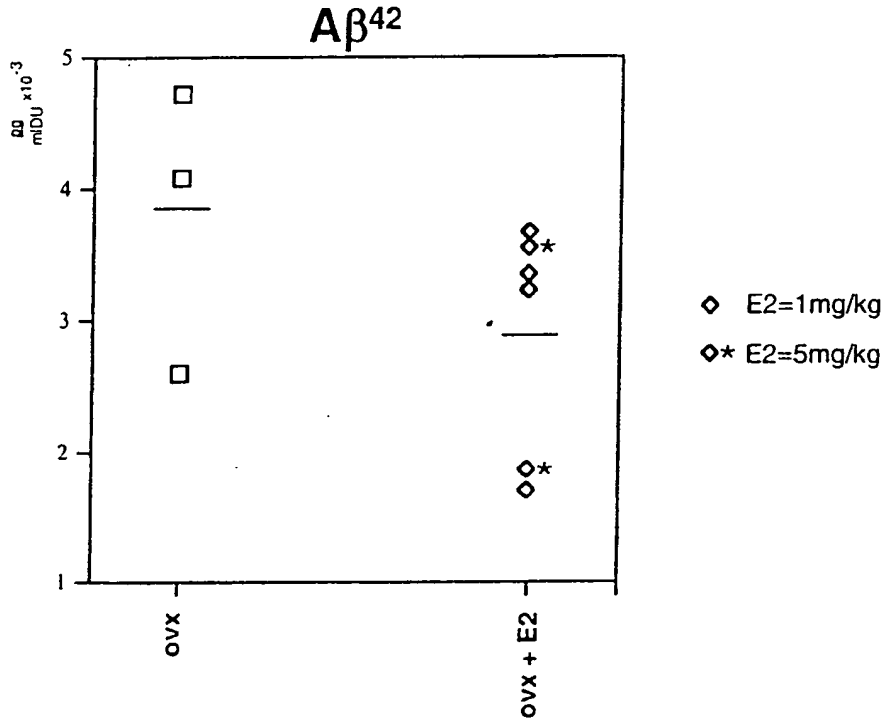

Aβ42
ng mlDU x10-3
5
4
3
2
1
OVX
OVX + E2
E2=1mg/kg
E2=5mg/kg

Figure 4

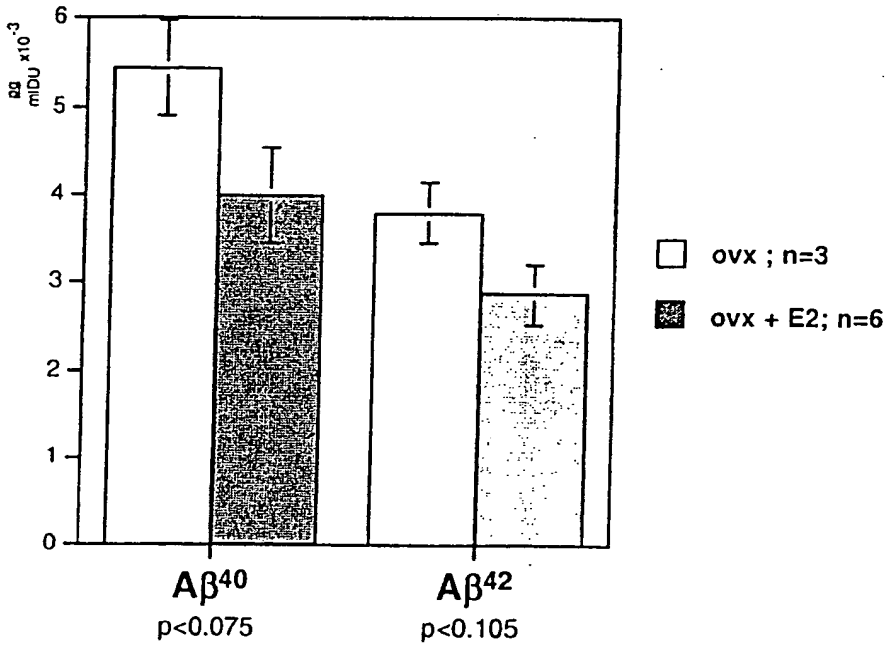
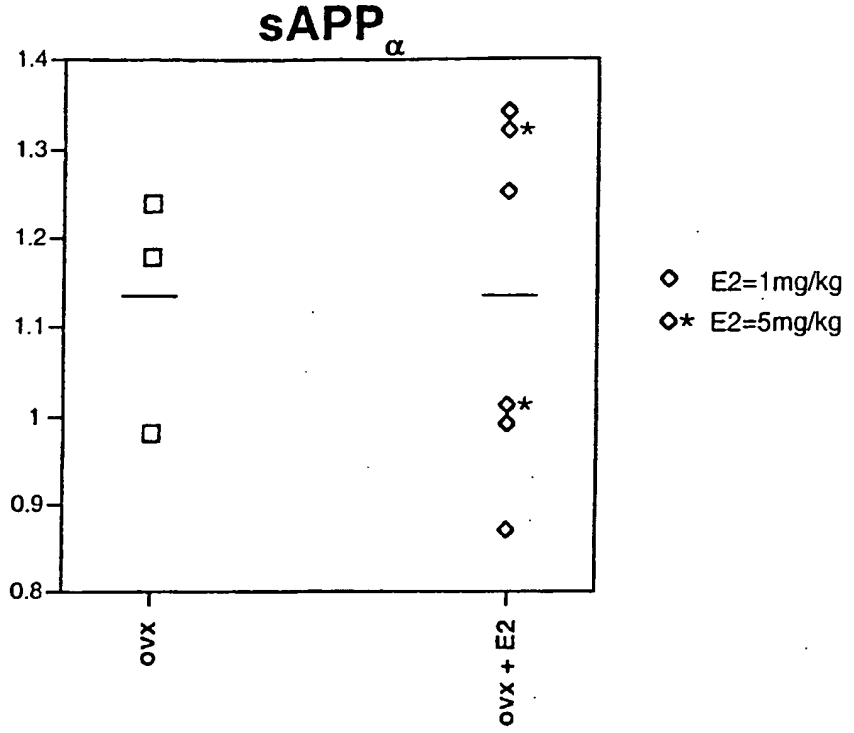
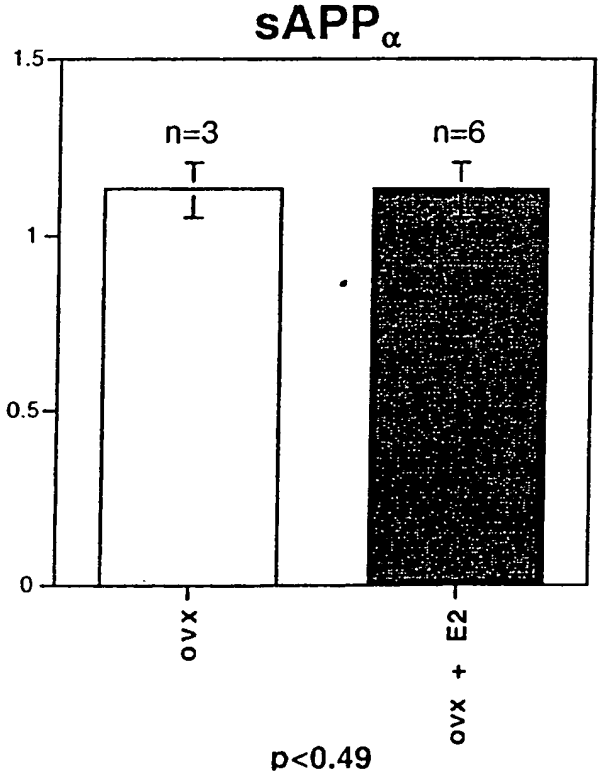


Figure 5

A.



B.



p<0.49

Redacted

Redacted

DRAFT

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by accumulation senile plaques in the brain, which are formed as a result of deposition of potentially neurotoxic, 40- and 42-amino acid amyloid β peptides (Aβ). Recent studies indicate that postmenopausal estrogen replacement therapy may prevent or delay the onset of AD (Tang et al., 1996). In addition, there is evidence that physiological concentrations of 17-β estradiol (E2) reduce the release of Aβ peptides from primary neuronal cultures of rat and mouse origin, and from human embryonic cerebrocortical neurons (Xu et al., 1998), suggesting a mechanism by which estrogen replacement therapy may delay and/or prevent AD.

Here we propose a methodology, which enables screening of the effects of estrogen on the levels of Aβ peptides in brain *in vivo*, using experimental animals (i.e., guinea pigs). We also present the results of a pilot study, which show that E2 can reduce the levels of Aβ peptides in brain *in vivo*. The proposed methodology will enable screening of the effects of short and long-term treatments with estrogen and estrogen-like compounds on the accumulation of Aβ peptides in brain *in vivo*.

Abbreviations: **AD,** Alzheimer's disease, **Aβ,** amyloid beta; **APP,** amyloid precursor protein; **flAPP,** full-length APP; **sAPP,** soluble APP; **ovx,** ovariectomized; **E2,** 17β-estradiol; **BW,** body weight; **UW,** uterine weight; **p.o.,** per os; **DEA,** diethyl amine; **FA,** formic acid; **ELISA,** enzyme-linked immunosorbent assay.

METHODS

Maintenance of animals and treatment. Nine ovariectomized female guinea pigs were purchased from Covance Research (Denver, PA); the animals were ~8 weeks old at the time of surgery. Throughout the study the animals were fed *ad libitum* in a controlled lighting environment (6 h light; 19 h dark cycles). During the first three weeks after surgery, the animals were fed standard lab chow (Purina, Richmond, IN); the standard lab chow was then replaced with powdered, casein-based, soy-free diet (catalogue # 5713M-O, Purina, Richmond, IN) to exclude the presence of phyto-steroids in the diet. After 5 weeks on soy-free diet the animals were divided into three experimental sets: i) ovx-only (n=3), ii) ovx+low E2 group, (n=4, 1mg E2/1kg BW), and iii) ovx+high E2 group, (n=2, 5mg of E2/1kg BW). *17-β estradiol* **(catalogue # E8875; Sigma, St. Louis, MO)** ***was administered p.o. by powdering the hormone into the soy-free chow.*** Prior to the beginning of the treatment, the animals were weighed, and average daily food intake for each animal using this particular diet was determined in order to supplement the hormone at the desired concentration. The ovx+E2 animals received estradiol for 10 days.

Tissue collection. At the end of the treatment, animals were sacrificed by decapitation. Trunk blood was collected for determination of E2 levels in the serum by radio-immuno-assay. Uteri were removed and weighed to establish estradiol-induced uterine hypertrophy. The brains were immediately dissected; the

cerebellum was removed from each brain, and the rest of the brain was divided in hemispheres, which were snap-frozen, and stored at -80°C.

Preparation of brain extracts. Soluble proteins from the brains were recovered using a modification of a previously described protocol (Savage et al., 1998). The hemispheres were homogenized in 0.2% DEA/50mM NaCl at 1:10 w/v ratio, by 5-6 strokes of a Dounce homogenizer. The DEA homogenate was centrifuged for 90 min at 100 000g. The DEA supernatants were neutralized to pH ~8.0 by addition of 1/10th vol. of 0.5M Tris-Cl pH 6.8, aliquoted and snap-frozen. The DEA pellets were homogenized in 70% formic acid, and centrifuged for 1 h at 100 000g. The FA supernatants were neutralized to ~pH 8.0 with 1N NaOH and multiple aliquots of each FA extract were snap-frozen. The protein concentrations of the DEA and FA supernatants were determined using the BCA reagent assay kit (Pierce, Rockford, IL).

Detection of sAPPα, flAPP, Aβ40 and Aβ42.

1) sAPPα was detected by western blotting of proteins from the DEA extracts using the monoclonal antibody 6E10 (Senetek, St. Loius, MO), which recognizes residues 5-10 from the Aβ region. The DEA extraction recovers soluble and not membrane embedded proteins; this was confirmed by probing duplicate western blots which contained increasing amounts of proteins from DEA extracts (10μg-200μg), with either 6E10, or an antibody against the

C-terminus of APP, C1.61 (courtesy of Dr. Paul Mathews, NKI) (Fig.1). Fig. 1 shows that soluble APP is the preponderant species in the DEA extract, while flAPP, albeit present, is a minor species.

For detection of the effect of E2 on the levels of sAPPα, we performed western blotting of duplicate samples from the DEA extracts (50μg/lane) with 6E10, followed by enhanced chemiluminescence. Multiple exposures of the immunoblots were scanned using the ScanAnalysis software. The average values (in densitometric units) for each sample were then normalized to the values obtained for flAPP. Full-length APP levels were determined by immunoblotting of FA extracts (20μg/lane) with antibody 369 (made against the cytoplasmic tail of APP, residues 645-695) in duplicate, and densitometric analysis of multiple exposures of the immunoblots.

2) The levels of soluble Aβ40 and Aβ42 were determined by Aβ40- and Aβ42-specific ELISA assays (Mehta et al. 1998) in the laboratory of Dr. Pankaj Mehta (Institute for basic Research in Developmental Disabilities, Staten Island NY), on a fee-for-service basis.

RESULTS

Summary: The 10-day oral administration of 17β estradiol, led to significant uterine hypertrophy in ovariectomized guinea pigs. This treatment also resulted in a decrease in brain Aβ40 and Aβ42 levels (~25%; this effect borders on statistical significance), but did not alter the levels of sAPPα. It is of note that we did not observe consistent dose-dependent changes in the affected parameters; except in the case of Aβ40.

Orally administered 17β estradiol causes uterine hypertrophy. In the course of 10 days the animals received either soy-free diet, or soy-free diet supplemented with E2 (1mg E2/1kg BW, or 5mg E2/kg BW). The uteri of the ovx+E2 animals were significantly hypertrophied, as shown on the scatter-graph in Fig. 2A. Comparing the uterine weight/body weight index for each animal showed the effect of E2 on the uterine weight. Fig. 2B is a bar-graph representation of the same data.

17β estradiol treatment shows a strong tendency for significant decrease of the levels of soluble, brain Aβ peptides, *in vivo*. E2 treatment resulted in a similar reduction of the levels of soluble Aβ40 and Aβ42 peptide. The scatter-graphs in Fig.3 show the values for Aβ40 (panel A.), and Aβ42 (panel B.) (pg/ml) normalized to the levels of flAPP (densitometric units. DU)

for individual animals. The bar-graph in Fig 4. depicts the mean values for Aβ40 and Aβ42 levels, for the ovx and ovx+E2 groups. The difference between the mean values of the ovx and ovx+E2 groups was 26.5%+/-13.3 ($p<0.075$) for Aβ40 and 24.3%+/- 14.0 ($p<0.105$) for Aβ42. It is important to note that at E2 dose of 5mg/kg, the Aβ40 levels were diminished by 43.1%+/-9.1% ($p< 0.03$).

Estradiol treatment does not alter the levels of sAPPα *in vivo*. The levels of sAPPα (determined by quantitative western blotting of extracts of soluble brain proteins) were not different between the ovx and ovx+E2 animals, as shown on the scatter-graph in Fig. 5A, and the bar-graph in Fig. 5B ($p<0.49$). The sAPPα values were normalized to the values for flAPP.

Figure 1

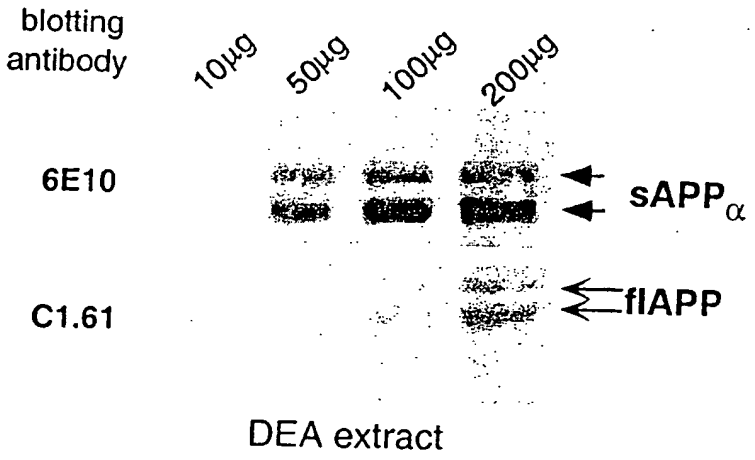
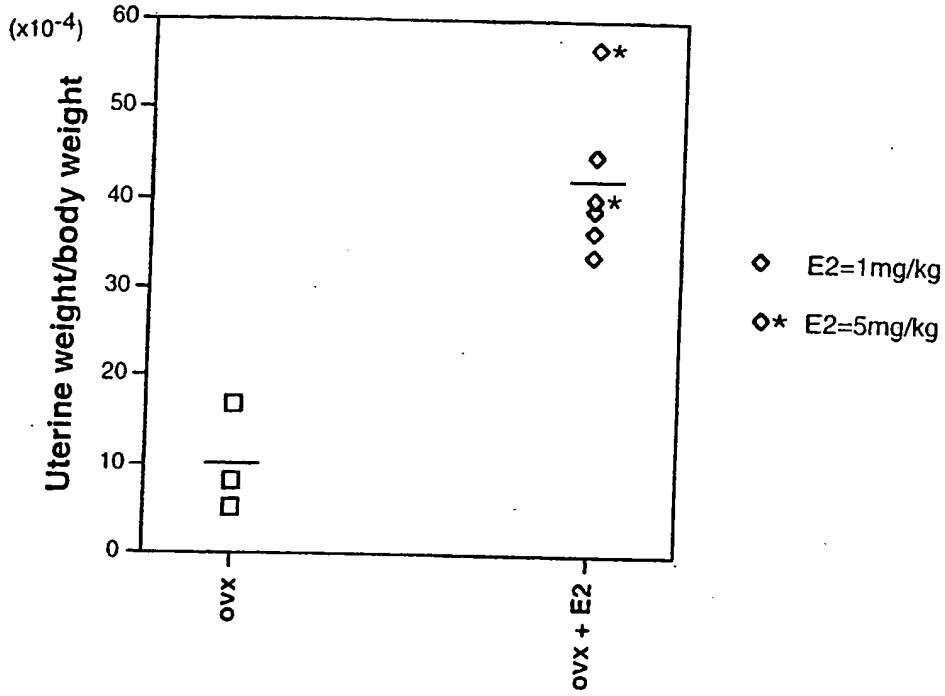

blotting antibody
10µg
50µg
100µg
200µg
6E10
C1.61
sAPP$_\alpha$
flAPP
DEA extract

Figure 2

A.


(x10-4)
60
50
40
30
20
10
0
Uterine weight/body weight
ovx
ovx + E2
E2=1mg/kg
E2=5mg/kg

B.

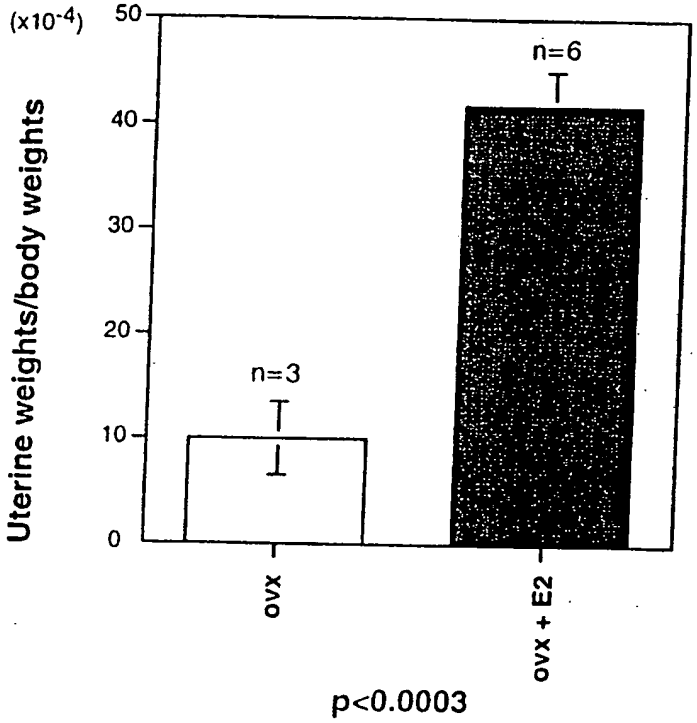
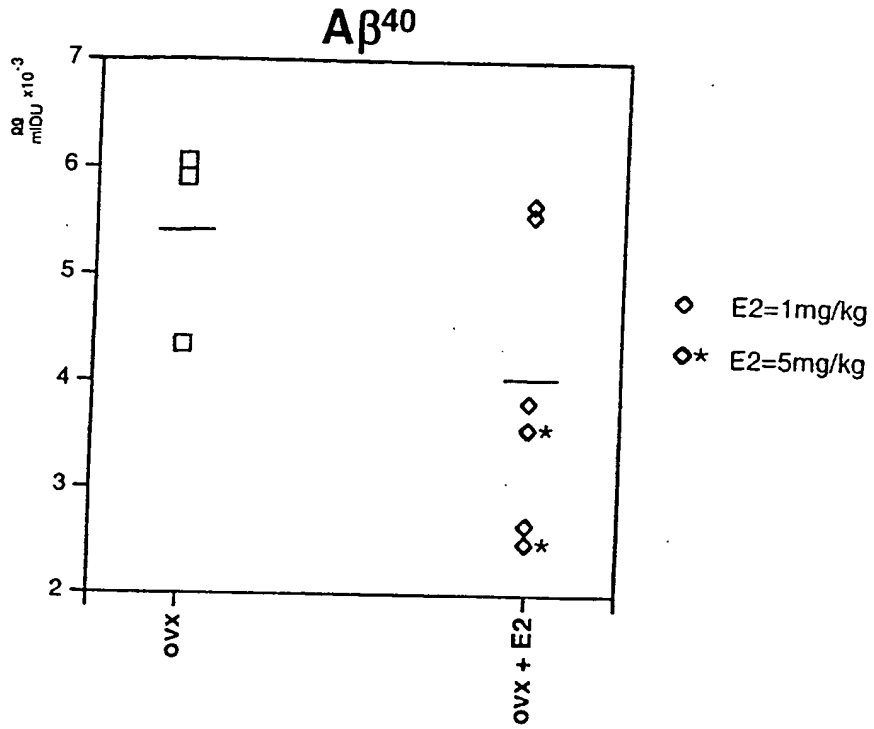

(x10-4)
50
40
30
20
10
0
Uterine weights/body weights
n=3
n=6
ovx
ovx + E2
p<0.0003

Figure 3

A.


Aβ40
log mIDU x10-3
7
6
5
4
3
2
OVX
OVX + E2
E2=1mg/kg
E2=5mg/kg

B.

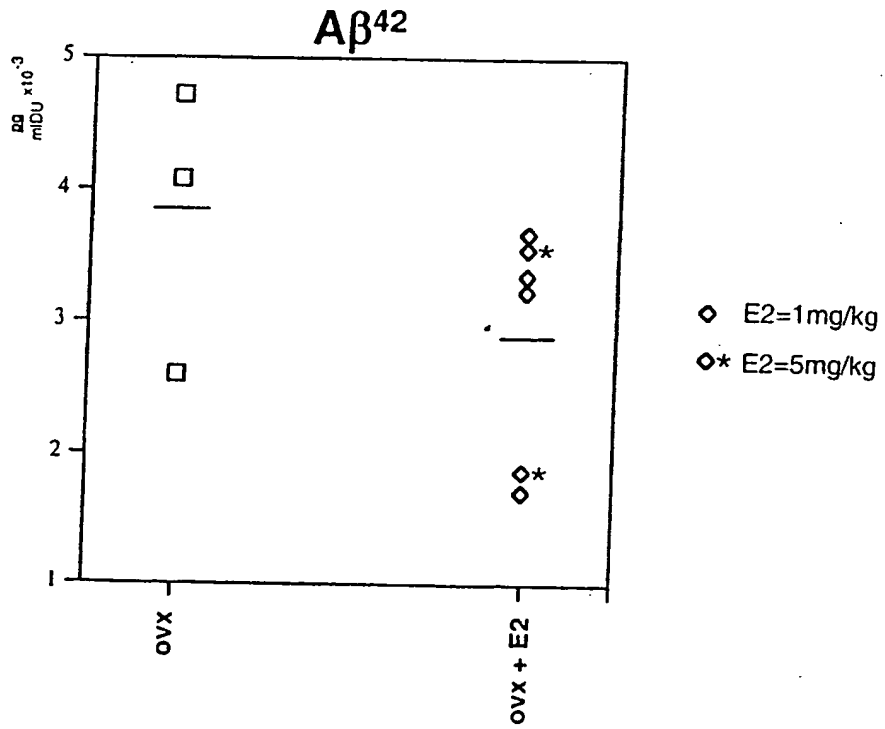

Aβ42
log mIDU x10-3
5
4
3
2
1
OVX
OVX + E2
E2=1mg/kg
E2=5mg/kg

Figure 4

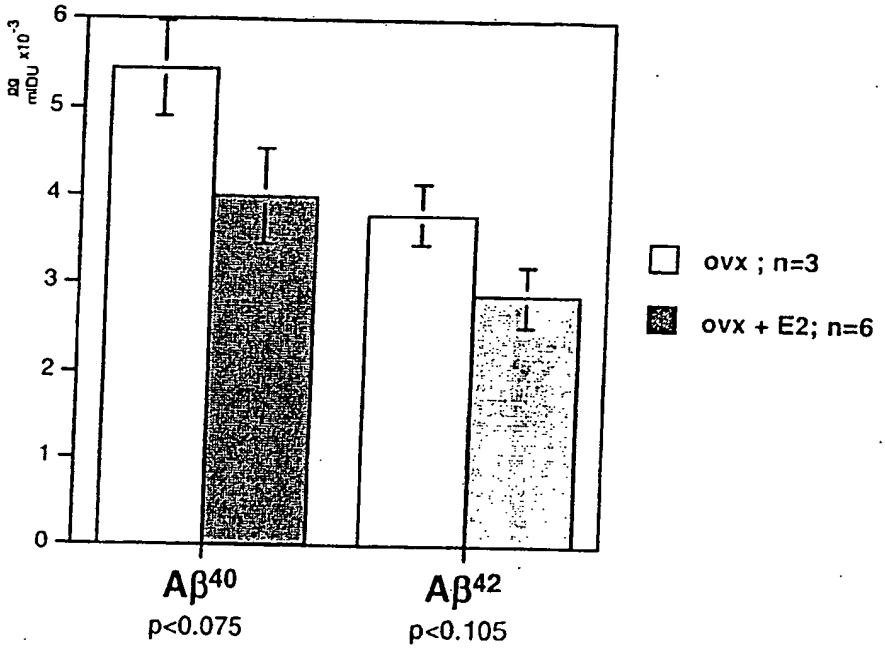
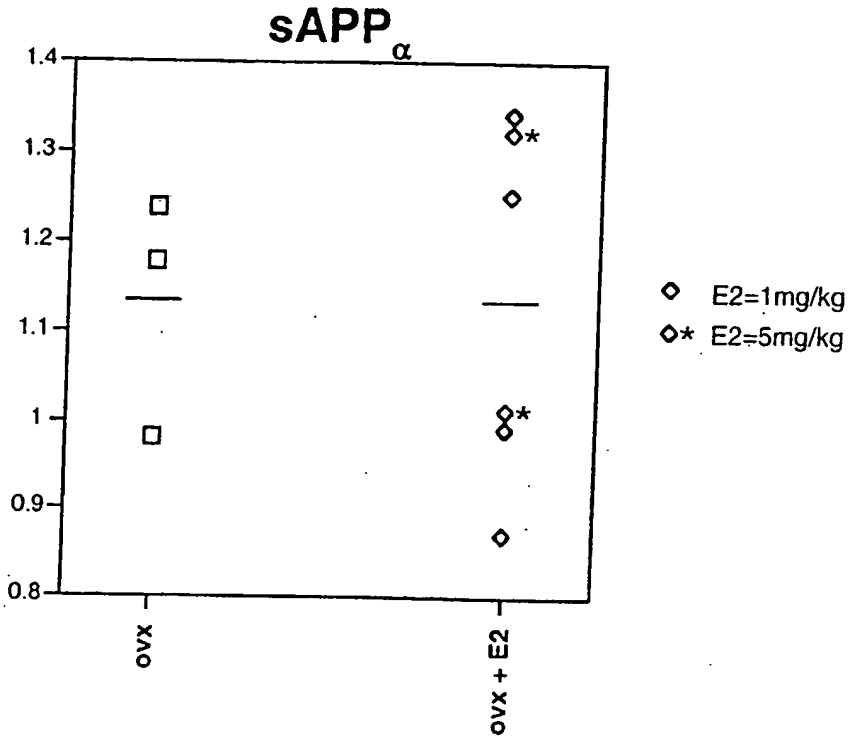
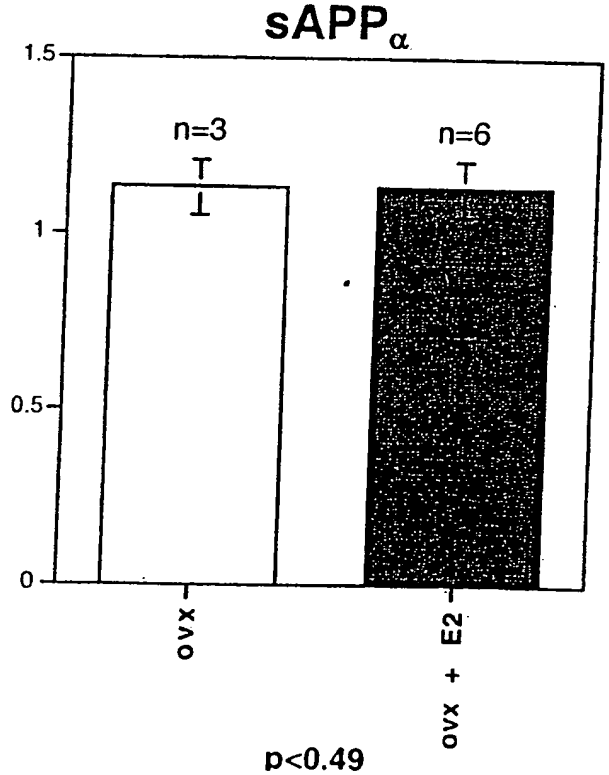


Figure 5

A.



B.



p<0.49

Molecular Brain Research, 10 (1991) 299–305

ADONIS 0169328X9170306S

BRESM 70306

Conservation of the sequence of the Alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis

E.M. Johnstone[1], M.O. Chaney[2], F.H. Norris[1], R. Pascual[3] and S.P. Little[1]

[1]Molecular Genetics Research and [2]Supercomputing Applications and Molecular Design, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285 (U.S.A.) and [3]University of California, Berkeley, CA 94720 (U.S.A.)

(Accepted 12 February 1991)

Key words: Alzheimer's disease; β-Amyloid; Cross-species; Polymerase chain reaction; Phylogenetic

Neuritic plaque and cerebrovascular amyloid deposits have been detected in the aged monkey, dog, and polar bear and have rarely been found in aged rodents (*Biochem. Biophy. Res. Commun.*, 12 (1984) 885–890; *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 4245–4249). To determine if the primary structure of the 42–43 residue amyloid peptide is conserved in species that accumulate plaques, the region of the amyloid precursor protein (APP) cDNA that encodes the peptide region was amplified by the polymerase chain reaction and sequenced. The deduced amino acid sequence was compared to those species where amyloid accumulation has not been detected. The DNA sequences of dog, polar bear, rabbit, cow, sheep, pig and guinea pig were compared and a phylogenetic tree was generated. We conclude that the amino acid sequence of dog and polar bear and other mammals which may form amyloid plaques is conserved and the species where amyloid has not been detected (mouse, rat) may be evolutionarily a distinct group. In addition, the predicted secondary structure of mouse and rat amyloid that differs from that of amyloid bearing species is its lack of propensity to form a β sheeted structure. Thus, a cross-species examination of the amyloid peptide may suggest what is essential for amyloid deposition.

INTRODUCTION

The presence of senile plaques represents one of the pathological lesions of Alzheimer's disease (AD) in humans. The major component of the plaque is β/A4, a 4-kDa peptide[9,20] derived from a larger precursor, amyloid precursor protein (APP). Aged humans, lower primates and other mammals normally develop neuritic plaque and cerebrovascular vascular amyloid deposits, yet rarely does the mouse or rat demonstrate this phenotype with age[24]. The neuritic plaques detected in the aged monkey, orangutan, polar bear and dog are observed in the absence of neurofibrillary tangles[25,28].

Southern blot analysis of genomic DNA using the APP cDNA as probe shows a clear sequence similarity among the human, mouse, hamster, rat, rabbit, sheep, guinea pig, dog, cow and chicken gene sequences[10,14,19,22,29]. Under stringent conditions, Northern blot analysis of RNA from brain tissue of human, rabbit, mouse, rat, hamster, guinea pig, dog, cow and chick[10,19] demonstrates that a similar sized messenger RNA (3.8 kb) and equivalent amount of APP RNA can be detected in eight mammalian species and in chicken. These observations suggest that there is conservation of both size and abundance of APP mRNA.

The mouse[32] and rat[27] cDNA sequence is about 90% homologous to the human cDNA. Three amino acid substitutions located in the β/A4 region distinguish the human sequence from that of mouse and rat yet these rodents rarely form amyloid plaques.

In the present study, we have made a detailed comparative analysis of the region of the β/A4 cDNA that encodes the amyloid peptide to not only provide more information about the primary protein sequence, but also to show the extent of silent mutations across species.

MATERIALS AND METHODS

Materials

A portion of polar bear brain was kindly provided by Prof. Linda Cork of Johns Hopkins University. Poly(A^+) RNA from canine kidney, rabbit brain, cow brain, sheep heart, pig brain and guinea pig brain was purchased from Clonetech Labs, Inc. All other reagents, materials and equipment are noted in their respective descriptions.

Correspondence: S.P. Little, Molecular Genetics Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, U.S.A.

RNA isolation and synthesis of first strand cDNA

Total RNA from polar bear brain was isolated by the acid phenol method of Chomczynski and Sacchi[2]. Briefly, a portion of frozen polar bear brain was placed in a solution of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, homogenized in a polytron (Brinkman Instruments) and extracted in phenol:chloroform:isoamylalcohol. RNA was precipitated in isopropanol.

Either 2.5 μg total RNA or 2.5 μg poly(A^+) RNA was reverse transcribed according to the method of Gerard et al.[8]. First strand cDNA was synthesized using either 0.01 μg/μl oligo$(dT)_{12-18}$ or 0.02 μg/μl random hexamers (Pharmacia) as primers in the presence of 0.1 μg/μl nuclease-free bovine serum albumin (BRL), 50 μg/ml actinomycin D, 0.5 mM dNTPs, 1 unit/μl RNAsin (Promega), 200 units MMLV reverse transcriptase and reverse transcriptase buffer (BRL). Reaction mixtures were incubated for 1 h at 37 °C, heated to 95 °C for 5 min, cooled on ice for 3 min and stored at 4 °C.

Polymerase chain reaction

PCR was performed essentially as described by Saiki et al.[23] in a Perkin-Elmer/Cetus Thermal Cycler. Between 0.5 and 2 μl of the first strand cDNA reaction mixture was amplified by PCR in 100 μl using 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus) and 20 pmol of each primer, EJ37-5' GGTTGACAAATATCAAGACGG 3' and EJ38-5' TGATGAATGGATGTGTACTG 3' (Research Genetics). To amplify cDNA sequence, we used conditions of 15 s at 96 °C (for denaturation), 30 s at 50 °C (to allow annealing of primer to template) and 1 min at 72 °C (for primer extension) for 35 cycles. The PCR products were analyzed on a 4% NuSieve (FMC), 1% SeaPlaque (FMC) agarose gel in TAE buffer. After ethidium bromide staining, the expected 229 bp fragment was excised, diluted into 1 ml water, and placed at 70 °C.

Asymmetric PCR and sequencing

Approximately 20 pg of the 229 bp fragments from each species were used for asymmetric PCR[11] using 0.5/50 and 50/0.5 pmol of primers EJ37 and EJ38 under the conditions described above. The products of these reactions were centrifuged twice through a Centricon-100 (Amicon) membrane, and 1/5th of the retentate was sequenced using TAQuence (United States Biochemical) with 1 pmol of the limiting primer from the respective asymmetric reaction. The primers were kinased for sequencing using γ-[^{32}P]ATP. First strand cDNA synthesis and asymmetric PCR of each species were performed at least two times with independent RNA preparations.

Phylogenetic tree

A phylogenetic tree was constructed using a comparison of 173 nucleotides of β/A4 from each species from the quality scores of the BESTFIT and WORDSEARCH programs of the UW Genetics Computer Group Software Package[5]. The distance between species was computed using a transformation of the quality scores. The algorithm DENDRON was used to compute the best (minimal standard deviation) phylogenetic tree[30].

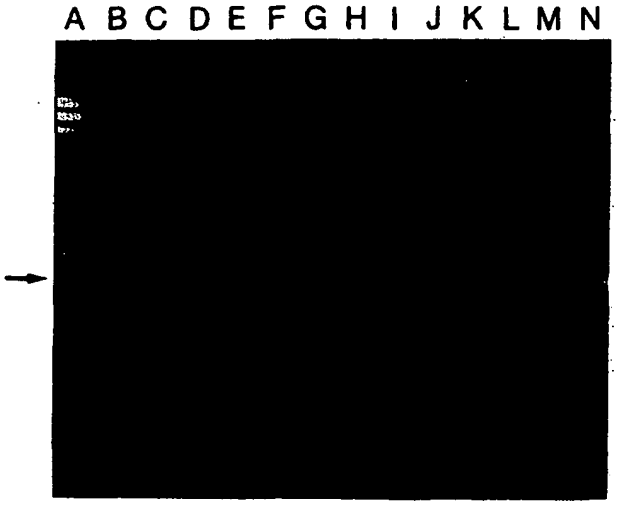


Fig. 1. Analysis of PCR-amplified cDNA. Lane A: Phi X 174 RI *Hae*III DNA marker. B–J: cDNA template generated from oligo(dT) primed RNA. K–N: hexamers as primer. B, dog; C, polar bear; D, rabbit; E, cow; F, sheep; G, pig; I, DNA marker; J, guinea pig; L, pig; M, guinea pig; N, pig. The arrow indicates the 229 bp product.

Predicted secondary structure

The α, β and turn propensities for the β/A4 peptide were calculated by the Chou Fasman secondary structure prediction algorithm[3].

RESULTS AND DISCUSSION

Cross-species PCR and sequencing

To assess similarities across species in β/A4 peptide sequence and length, we PCR amplified cDNA prepared from a variety of sources. Total RNA from frozen polar bear brain and poly(A^+) RNA from dog, rabbit, cow, sheep, pig and guinea pig were used in our experiments. Although the dog and sheep RNA was not derived from the brain, we have assumed that these species have only one APP gene.

TABLE I

Matrix of distances between species of the β/A4 coding region

	Cow	*Dog*	*Guinea pig*	*Human*	*Mouse*	*Pig*	*Polar bear*	*Rabbit*	*Rat*	*Sheep*
Cow	0.0	19.2	27.2	12.8	30.4	28.8	24.0	24.0	30.4	6.4
Dog	19.2	0.0	25.6	12.8	32.0	24.0	11.2	16.0	24.0	16.0
Guinea pig	27.2	25.6	0.0	17.6	28.8	33.6	32.0	28.8	27.2	27.2
Human	12.9	12.8	17.6	0.0	30.4	25.6	14.4	16.0	27.2	9.6
Mouse	30.4	32.0	28.8	30.4	0.0	36.8	41.6	33.6	20.8	28.8
Pig	28.8	24.0	33.6	25.6	36.8	0.0	32.0	22.4	24.0	24.0
Polar bear	24.0	11.2	32.0	14.4	41.6	32.0	0.0	24.0	30.4	20.8
Rabbit	24.0	16.0	28.8	16.0	33.6	22.4	24.0	0.0	27.2	19.2
Rat	30.4	24.0	27.2	27.2	20.8	24.0	30.4	27.2	0.0	27.2
Sheep	6.4	16.0	27.2	9.6	28.8	24.0	20.8	19.2	27.2	0.0

```
                    S  E  V   K  M [D  A   E  F  R   H  D  S   G  Y  E  V
Human(3)   1773 CTCTGAAGTG AAGATGGATG CAGAATTCCG ACATGACTCA GGATATGAAG
Dog             ...C..G... .......... .C..G..... .......... ..........
Polar bear      ......G... ........C. .C..G..... .......... .....C....
Rabbit          ...C...... ..A....... .G..G..... ......T..T ........G.
Cow             .......... .......... ..:....... ......T... ..........
Sheep           .......... .......... ..:....... ......T..C ..........
Pig             .......... .......... .G..G..... ...C..T..G ..C.....G.
Guinea Pig      ...G.....A .......... .......... .......... ..........
Mouse (5)       ...G...... .......... ........G. ......T... ....T.....
Rat(6)          ...A...... .......... .G..G...G. ......T... ..C.TC....
                                                 *G              *F

                    H  H  Q   K  L  V   F  F  A  E   D  V  G   S  N  K
Human      1823 TTCATCATCA AAAATTGGTG TTCTTTGCAG AAGATGTGGG TTCAAACAAA
Dog             .......... .......... .......... .......... C.........
Polar bear      .......... G......... .......... .......... C.........
Rabbit          .......... .......... ........T. .......... ..........
Cow             .......... .......... .......... ....C..... ...C......
Sheep           .......... .......... .......... .......... ...C......
Pig             .......C.. ....C..... .....C.... .......... ..........
Guinea Pig      .C........ ....C..... .......... .......... ..........
Mouse           .C.GC..... ....C..... ........T. .......... ...G......
Rat             .C.GC..... ....C..... .......... .......... ..........
                 *R

                    G  A  I  I   G  L  M   V  G  G   V  V  I  A]  T  V  I
Human      1873 GGTGCAATCA TTGGACTCAT GGTGGGCGGT GTTGTCATAG CGACAGTGAT
Dog             .....C.... .......... .......... .......... .A........
Polar bear      .....C.... .......... .......... .......... ..........
Rabbit          .....G.... .......... .......... .......... ..........
Cow             ..A..C.... ....G..... .......... .......... .A........
Sheep           .....C.... ....G..... .......... .......... .A........
Pig             .....C.... .......... ......T... .......... .A..C.....
Guinea Pig      ........T. .C........ ...C..T..C .......... .A........
Mouse           ..C..C.... .C........ .........C .......... .A..C.....
Rat             .....C.... .......... ......T..C .......... .A........

                    V  I  T   L  V  M  L
Human      1923 CGTCATCACC TTGGTGATGC TGA
Dog             T......... .......... ...    8
Polar bear      .......... .....A.... ...    9
Rabbit          T......... .......... ...   10
Cow             .........T .......... ...    8
Sheep           .......... .......... ...    6
Pig             T......... ..A....... ...   16
Guinea Pig      T........T .......... ...   12
Mouse           T......... C........T ...   19
Rat             T......... .......... ...   17
```

Fig. 2. Homology of β/A4 cDNA. Nucleotides are numbered according to Kang[14]. Conserved nucleotides are indicated by dots. The total number of base changes, as compared to human is indicated after each sequence. The deduced amino acid sequence is indicated above the nucleotide sequence. *G, *F, *R indicate mouse and rat amino acid changes from human[2,27]; square brackets ([ ]) indicate 42 residue β/A4.

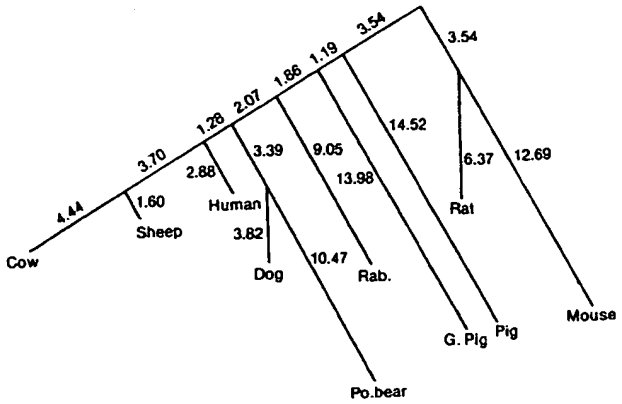


Fig. 3. Phylogenetic tree representing a comparison of 173 nucleotides of each species. The shortest distance between two species indicates a cluster. The distance between a pair of species is the sum of the branch lengths.

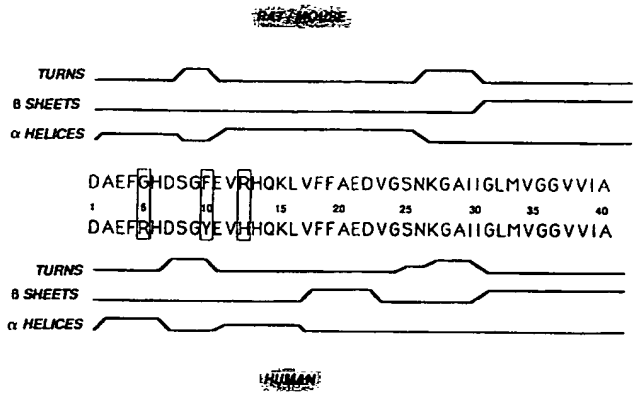


Fig. 4. β sheet and turn propensities for the β/A4 peptide as calculated by the Chou and Fasman[3] prediction of protein secondary structure. The human amino acid sequence is identical to the monkey[21], polar bear and dog sequence.

PCR primers representing nucleotides 1745–1765 (EJ37) and 1954–1973 (EJ38) of human APP as described by Kang et al.[14] were designed to copy homologous sequences of the various species of mammals. The mouse cDNA sequence is identical to the human sequence in the region of the primers.

Complementary DNA synthesized using oligo(dT) as primer was used successfully as template for PCR products of the polar bear, dog, rabbit, cow, sheep and pig, as seen in Fig. 1. A PCR product of similar size was generated from these cDNAs and suggested a conservation of length of the coding region for the β/A4 peptide. Several unsuccessful attempts were made to detect a PCR product generated from the oligo(dT) primed guinea pig poly(A^+) RNA (lanes H and J). In the event that the lack of PCR product was due to secondary structure, length or scarcity of template, we used random hexamers as primers for the reverse transcriptase reaction. The results are shown in Fig 1, lanes K–N.

An annealing temperature of 50 °C for 30 s was adequate to generate detectable PCR products from all species tested. Each cross-species PCR analysis represents at least two separate cDNA reactions. PCR products migrating at 229 bp were used as template for subsequent sequencing reactions.

One concern with PCR amplification of cDNA for sequence analysis is the potential for errors introduced during the amplification process. Instead of cloning and sequencing several clones, we performed asymmetric PCR using the initial PCR product as template. Here sequences incorrectly copied represent only a small fraction of the pool of PCR products.

The nucleotide sequence representing 173 bp from the various mammalian species is displayed in Fig. 2 along with the published human, mouse, and rat sequence. The cDNA sequence for monkey β/A4 was not included in our analysis[21]. Comparison of the deduced amino acid sequence of human, dog, polar bear (plaque bearing species) as well as rabbit, cow, sheep, pig and guinea pig shows identity. Only the mouse and rat sequences contain differences[27,32]. The total number of nucleotide differences from human sequence is noted at the end of each sequence. In comparison with the human sequence, the greatest percentage of silent mutations in this region of the APP cDNA was in the pig sequence (28.0%) followed by mouse (24.0%), rat (22.8%), guinea pig (21.0%), rabbit (17.5%), polar bear (15.7%), dog and cow (14%) and sheep (10.5%). It appears that the nucleotide differences were not distributed randomly throughout the amyloid peptide (43 residues). Most noticeably, aspartic acid no. 7 and alanine no. 31 of the β/A4 peptide demonstrate this type of conservation. These residues are in the vicinity of the predicted turn regions of β/A4 secondary structure (Fig. 4).

Predictions made from nucleic acid sequence

By assessing β/A4 mutations that result in amino acid changes across species, we may elucidate the nature of amyloid plaque deposition as well as the structural and functional relationship of the domains of the β/A4 peptide. Primate β/A4 peptide sequence is identical to human[21]. The three recorded sites of mutation in mouse and rat in β/A4 at residues 5, 10 and 13 are all within the region of the APP polypeptide proposed to be extracellular[14] and hydrophillic. These mutations occur within one of the two exons that encode the β/A4 peptide[13,16,33]. Since a mutation can effect the function or structure of a domain, perhaps this hydrophillic domain may control cerebral plaque formation and hence mice/rats do not form condensed plaques.

Mutations in the hydrophillic region (residues 88–97) immediately adjacent to the hydrophobic domain of

prion amyloid may explain the presence (in Syrian hamster, Chinese hamster and VM mice) and absence of plaque (in Armenian hamster) in scrapie pathology[18]. The only known human mutation that affects β/A4 deposition is the glutamic acid to glutamine substitution at residue 22 of the β/A4 in hereditary cerebral hemorrhage (Dutch Type) (HCGWA-D) (25). HCHWA-D patients develop amyloid deposits in the vessel walls of the leptomeninges and cerebral cortex. These deposits resemble the immature or diffuse plaque rather than the mature senile plaque of AD. The HCHWA-D mutation appears in the second exon of the β/A4 peptide[13] and may therefore reflect a function or structure involved in deposition of amyloid as a diffuse plaque.

Estimation of phylogeny

We have examined the phylogenetic relationships between ten species by comparing a 173 bp sequence encoding the β/A4 peptide. The calculations from DNA similarity programs can be true indications of similarity between sequences[4,26]. The UWGCG computer programs BESTFIT and WORDSEARCH produce an optimized score indicating the quality of the similarity between sequences. This quality score can be transformed into a measure of distance, is metric, and can be used as input to clustering programs.

The quality scores generated from WORDSEARCH were converted to distances by subtracting each from the maximum possible quality score (173) (Table I). These distances were used as input data to cluster and produce a tree diagram of the distances between these genes using an algorithm; DENDRON[30] (Fig. 3). As a check, the phylogenetic package PHYLIP by Felsenstein[6,7], which computes evolutionary distances, was used and produced a tree which matched closely the DENDRON tree.

The phylogenetic tree displayed in Fig. 3 shows that the mouse and rat sequences are most divergent from those species that do accumulate amyloid plaques (human, dog and polar). We predict that aged cows and sheep may accumulate plaques because they are clustered with human, dog and polar bear. We would have predicted that the guinea pig β/A4 sequence would have clustered with mouse and rat sequences because they are all rodents, but the guinea pig nucleic acid sequence is more related to the human sequence and is identical to the human amino acid sequence. In a search to identify a relevant and practical model for amyloid deposition, the guinea pig may provide a model for some aspects of AD.

Predicted secondary structure of human and rat/mouse β/A4

Because fibrillar β/A4 amyloid isolated from the AD brain (neuritic amyloid plaque), as well as synthetic β/A4 fragments under certain conditions have been shown to exhibit anti-parallel β-sheet secondary structure and cross-β supersecondary structure[15], fibril formation may be associated with β/A4's ability to form β-sheets. Hollosi et al.[12] have shown by CD measurements that human synthetic β/A4 (1–12, 11–25 and 1–28) fragments exhibit different conformation in polar and non-polar solvents. In an aqueous environment, a 'pleating process' takes place within the β/A4 (1–28) central segment (between residues 11 and 25) that may initiate and stabilize its observed β-sheet structure and eventual fibril formation.

The method of Chou and Fasman[3] was used to determine whether potential differences in predicted secondary structure existed between the human (monkey, dog, polar bear) and rodent (rat, mouse) β/A4 peptide. These results are compared in Fig. 4. Both forms exhibited a common β-sheet region between residues 30 and 42, and an α-helical structure from residue 1 to 7; however, marked differences were observed within the 11–25 region. Human β/A4 showed a propensity to form a β-sheet structure between residues 16–23, while the rodent exhibited a strong tendency to form an α-helical structure within the 11–25 region. These observations suggest that human β/A4 is more likely to form an anti-parallel β-sheet structure with the 30–42 predicted β-sheet region serving as a 'nucleation site' for inducing the 16–23 and 1–7 regions to form another anti-parallel β-sheet. The resulting conformation is stabilized as a cross-β supersecondary structure. The rodent, however, due to its predicted 11–25 α-helical region, blocks further propogation of β-sheet regions and cross-β structures and as a consequence may not form a fibrillar β/A4 structure.

Knowledge of the location and type of amino acid changes in the rodent sequence provides us with further information besides how they influence ordered structure. If rodents do not exhibit in vivo plaques because they possess less β-sheet structure compared to human and β-sheet conformations are necessary for fibril and plaque formation, then knowledge of where these rodent-human mutations occur may define specific molecular therapeutic targets for fibril destabilization.

SUMMARY

We have shown that the amino acid sequence of the β/A4 peptide in dog and polar bear is identical to the human and monkey sequence. These species all accumulate amyloid plaques with age. We have also shown that the amino acid sequence of rabbit, cow, sheep, pig and guinea pig is identical to the human sequence. Whether these species deposit amyloid during aging is not known. Overall, the deduced amino acid sequence and cDNA

sequence of ten species of amyloid peptide suggests a strong conservation of these residues as a functional domain, for example as peptide hormone[1,31] or an essential structure in APP maturation and processing.

We have also shown that there is greater evolutionary distance, as measured by nucleotide similarities, between those groups of species in which amyloid deposits have and have not been found than within these groups themselves. This same relationship holds between those groups in which the amino acid sequence of the β/A4 peptide is and is not conserved. In addition to this, the predicted secondary structure of β/A4 of humans suggests that a region between residue 12–28, predicted to form β-pleated sheet, may be important for amyloid deposition. The mouse and rat predicted structure is different in this region and could explain why these species do not form β/A4 mature plaques.

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